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TITLE: Regulation of ErbB Signaling in Breast Cancer Epithelial Cells by Cbl Proto-Oncogene Product

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13. ABSTRACT (Maximum 200 Words) Our laboratory investigates the mechanism of action of Cbl, a protein that is inducibly recruited to activated EGF receptors (EGF-R) and enhances receptor ubiquitination, downregulation, and degradation. Understanding the molecular basis for EGF-R regulation is an important goal in breast cancer research: the receptor is deregulated in up to 48% of all breast cancers. By defining the mechanisms used by Cbl to attenuate EGF-R signaling, we may identify interventions for the treatment of breast cancers. We have proposed to determine whether the recruitment of Cbl to EGF-R is ligand-selective and critical for the termination of signaling by the receptor. In linked studies of receptor signaling and subcellular localization, we will determine whether different EGF-R ligands that elicit distinct biological responses do so because of their differential recruitment of Cbl to EGF-R. In the 8.5 months since the award was reactivated, we have established the experimental parameters necessary to complete Tasks 1, 5, and 6. A key finding of this work is that Cbl is recruited equally well to EGF-R activated by EGF or TGF-alpha, and Cbl ubiquitinates the differently activated receptors identically. This indicates that the recruitment of Cbl to EGF-R is not sufficient to target the protein for degradation.					
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## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	3
Body.....	4-9
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusions.....	12
References.....	—
Appendices.....	13

## Introduction

A large body of evidence has shown that members of the epidermal growth factor (EGF) family play critical roles in the proliferation and differentiation of normal breast epithelial cells and are important for cell proliferation in breast cancer. The response to EGF is mediated by the transmembrane receptor tyrosine ErbB1, or epidermal growth factor receptor (EGF-R). This receptor plays a key role in the induction of proliferation in mammary epithelial cells (MECs). Overexpression of EGF-R due to gene amplification or increased transcription is seen in a third or more of all breast cancers. Receptor overexpression may be causally linked to oncogenesis, and correlates with development and progression of tumors that are refractile to standard therapeutic approaches. Thus, EGF-R is a potential target for therapeutic intervention in the subset of women with worst prognosis on conventional treatment. Understanding and manipulating the biochemical mechanisms regulating signal transduction through EGF-R therefore represent important goals in breast cancer research, and are the focus of this study. We have proposed the hypothesis that the proto-oncoprotein Cbl provides ligand-specific negative regulation of EGF-R in epithelial cells. We and others have shown that Cbl effects EGF-mediated down-regulation, ubiquitination, and degradation of EGF-R. In the 8.5 months of this reporting period (following reactivation of the award at the place of the P.I.'s independent appointment), we have tested whether Cbl's recruitment to the receptor and its ability to ubiquitinate EGF-R depends on the nature of the stimulating ligand. *In vivo*, EGF stimulation of EGF-R leads to receptor downregulation and the termination of signaling, but TGF- $\alpha$  stimulation of EGF-R leads to receptor recycling and prolonged signaling. We have found that EGF-activated EGF-R binds to Cbl and undergoes polyubiquitination, but TGF- $\alpha$ -activated EGF-R does the same. Thus, the recruitment of Cbl to EGF-R and its ubiquitination of the receptor is not sufficient to target the protein for inactivation and destruction. Other ligand-specified mechanisms are required for this process, and they may be Cbl-dependent. Ongoing studies address this issue.

## Body

This report summarizes research progress accomplished in a total of 8.5 months of Grant Years 2 and 3, months 24-31 of my Career Development Award. At the end of month 23 (on March 31, 2000), the award was suspended at my request in anticipation of a change in grantee institution. I now hold an appointment as an Assistant Professor of Pharmacology at the University of Iowa. To satisfy my reporting obligation and to facilitate reactivation of the award, I submitted a Progress Report in May 2001 covering the 11 months of Year 2 that had been funded prior to suspension of the award. This report covers progress made from September 2001- May 2002.

**Task 1:** Two attempts to generate mammary cell line transfectants overexpressing wild type or mutant Cbl proteins have been unsuccessful. The overexpression of Cbl inhibits mammary epithelial cell growth under specific culture conditions, potentially via induction of cell cycle arrest or apoptosis. Because mammary epithelial cells are poor substrates for transient transfection, it has been necessary to continue the proposed studies using the human embryonic kidney (HEK) 293 cell line. Like mammary epithelial cells, HEK 293 cells are of epithelial origin. They are easily transfected to express physiologically relevant amounts of EGF receptor (EGF-R) and Cbl proteins, and were the target cells used for optimization of the EGF-R downregulation assay developed for this work. In our approved Statement of Work, we proposed to use these cells as well as mammary epithelial cells. By optimizing our experimental assays on the HEK 293 cells, we are ready to proceed with experiments in the mammary epithelial cells as soon as we have derived them. We will continue to attempt to produce stable expressor mammary epithelial cell lines, but we will also initiate work to develop inducible mammary epithelial cell lines whose continuous growth in culture will not be compromised by constitutive Cbl overexpression. For this work, we will use the pREVTet-off system. A parental inducer mammary epithelial cell line will be derived that will be activated in the absence of doxycycline from the cell culture media. The inducer line will be used for infection by replication-defective retroviruses encoding the Cbl proteins of interest. Transduced cells will be selected on the basis of antibiotic resistance conferred by the Cbl-encoding plasmid. Clonal inducible expressor lines will be derived and utilized in studies detailed in Tasks 1-6 of the Statement of Work.

Results reported by us and the Yarden laboratory showed that Cbl negatively regulates EGF-R signaling by enhancing the down-regulation, ubiquitination, and degradation of ligand-activated receptors. Cbl is an E3 (protein-ubiquitin ligase) mediating target protein ubiquitination. Using an *in vitro* approach, three groups showed that Cbl enhances the linkage of ubiquitin moieties to a number of

receptors, including EGF-R. These results seem to suggest that Cbl negatively regulates receptor signaling by enhancing ubiquitin-dependent receptor degradation. However, the mechanism of Cbl-mediated receptor regulation requires further clarification.

**Task 1 Accomplishments (months 24-30).** We optimized the Task 1 experimental system in the HEK 293 system approved for use in Task 4 of the Statement of Work. We have determined that the Cbl's recruitment to ligand-activated EGF receptor is not ligand-selective (Task 1). EGF-activated EGF-R binds to Cbl and undergoes polyubiquitination, but TGF-alpha-activated EGF-R does the same (Fig. 1, below). Notably, only EGF-activated EGF-R is programmed for inactivation and degradation; TGF-alpha-activated EGF-R is recycled and its signaling is prolonged. Thus, the recruitment of Cbl to EGF-R and its ubiquitination of the receptor are not sufficient to target EGF-R for inactivation and destruction. Other ligand-specified mechanisms are required for this process, and they may be Cbl-dependent.

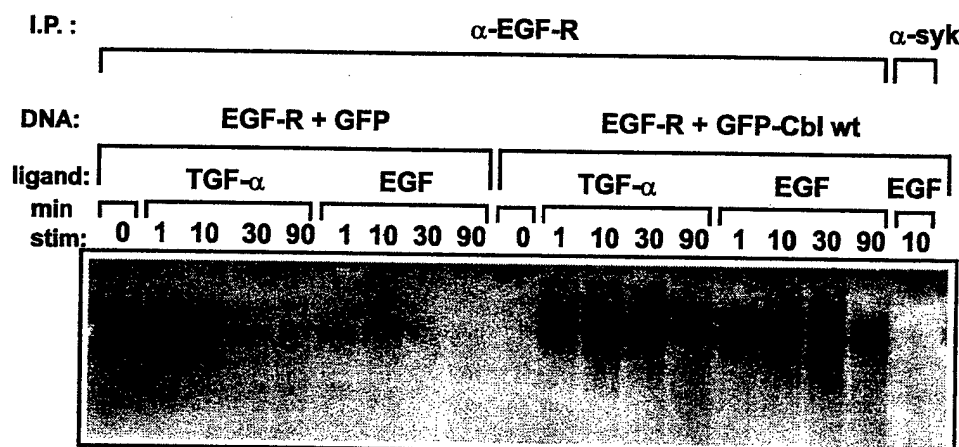


Fig. 1 TGF-alpha and EGF stimulation similarly enhance EGF receptor ubiquitination. Transfected HEK 293 cells were serum-starved for 4 h prior to stimulation with the indicated ligands (17 nM). The stimulated cells and unstimulated controls were lysed in 0.5% Triton X-100

lysis buffer. Lysates were clarified and protein levels quantitated. Equal amounts of protein from all lysates were subjected to anti-EGF-R immunoprecipitation. The collected immunoprecipitate proteins were gel-resolved, transferred to PVDF membrane, and immunoblotted with an antibody (NCL-UBIQ) specifically recognizing ubiquitin. Following incubation with an HRP-conjugated secondary reagent, the filter was processed for ECL detection of specific antibody binding.

We also have determined that EGF-R activated by EGF vs. TGF-alpha is comparably phosphorylated on tyrosine residues (Task 1; not shown). Again, similar results induced by the two ligands argues that the induction of differential receptor phosphorylation is not the key to the divergent biological impacts of these ligands.

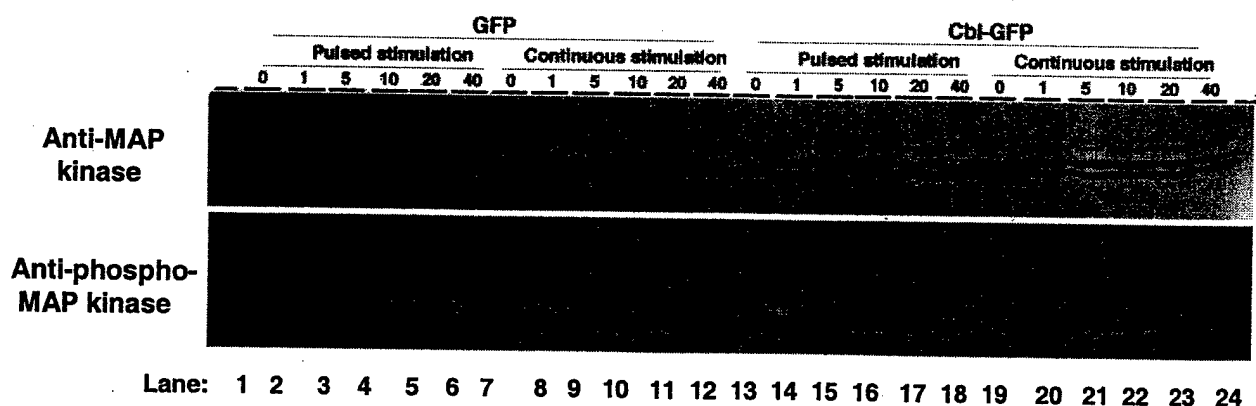
**Task 2 Accomplishments: completed in years 1-2. See Progress Report of May 2001.**

**Task 3 (months 31-40):** This Task assesses the effect of Cbl proteins on the mitogenic response induced by different EGF-R ligands. These studies can be undertaken only upon derivation of the stable or inducible Cbl-expressor lines. We plan to undertake this work within the next 6 months, as originally scheduled in the Statement of Work.

**Task 4 (months 36-40).** Progress on Task 4 partially requires the inducible or stable expressor cell lines overexpressing Cbl. This Task is designed to determine the tyrosine kinase activity of the EGF-R in cells expressing wt or mutant Cbl proteins. The approved Statement of Work indicated that these studies would be performed using transiently transfected HEK 293 cells and stable expressor mammary epithelial cells. According to the original Statement of Work, we will undertake these studies over the next year.

**Task 5 (months 36-48):** The purpose of this Task is to assess the nature of signaling events downstream of EGF-R in Cbl-transfected epithelial cells. In months 24-30, we have optimized our experimental techniques using transfected HEK 293 cells.

One signaling cascade functioning downstream of activated EGF-R is the MAP kinase signaling pathway. We assessed the intensity and duration of MAP kinase (Erk 1/2) activation following ligand stimulation of EGF-R in cells overexpressing Cbl or an epitope tag control. Preliminary results suggest that overexpression of Cbl both accelerates and abbreviates MAP kinase activity following EGF-R stimulation (Fig. 2).



**FIG. 2** Expression of wild type Cbl accelerates both MAP kinase activation and attenuation. HEK 293 cells transfected to express EGF-R and the indicated GFP-Cbl proteins were stimulated with EGF for the times shown and then lysed in 0.5% Triton X-100 extraction buffer. 100 µg of protein from each postnuclear supernatant was gel-resolved, transferred to a PVDF membrane, and probed using antibodies specific to activated (anti-phospho-MAP-K) or all forms of p44/p42.

Companion studies on the PLC gamma-1 signaling pathway have revealed that Cbl overexpression does not appear to alter PLC activation. However, another report indicates that in T cells, overexpression of Cbl suppresses PLC activity but not PLC activation. Our data are consistent with this report (Fig. 3). Additional signaling studies will be undertaken within the next year, as proposed in the approved Statement of Work.

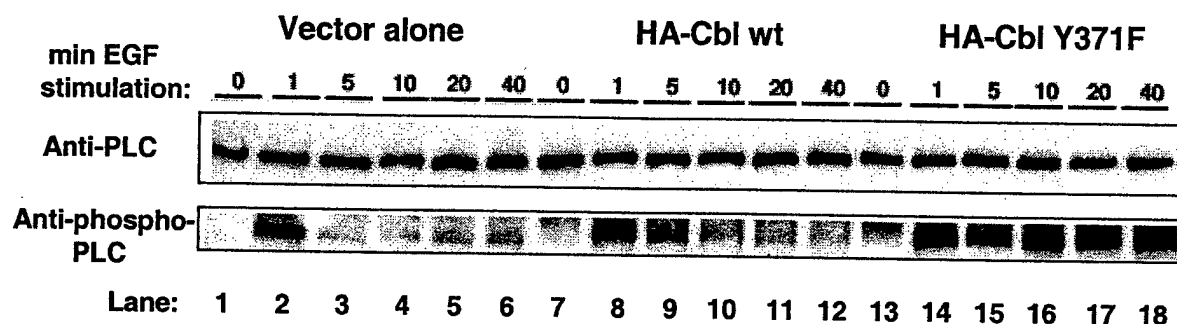


FIG. 11 Expression of dominant negative (Y371F), but not wild type, Cbl prolongs PLC- $\gamma$  activation. HEK 293 cells were processed as described in Fig. 2. Lysate proteins were probed with antibodies specific for activated (anti-phospho-PLC) or all forms of PLC- $\gamma$ .

Because our genetic analysis has shown that many Cbl-cellular protein interactions implicated in EGF-R signaling are not involved in negative regulation of the receptor, a revised approach is indicated for funding Years 3 and 4 to identify interactions that are important. The proposed experiments are outlined in the revised research plan.

**Original Task 6 (months 36-48).** The purpose of this task is to assess the effect of transfected Cbl on the level and subcellular localization of EGF receptor. **As described in the previous year's progress report, this task is largely completed.** We have continued these studies to identify organelles at which Cbl may regulate EGF-R location. That work also will be completed over the next year, according to the approved Statement of Work.

**In summary,** Tasks 2, 5, and 6 have been largely addressed within the first three years of the funding period. These experiments have resulted in the publication of a first-author manuscript and a second-author manuscript, and in the drafts for two last-author manuscripts to be submitted for publication shortly. Another outcome of the work has been the appointment of the principal investigator as an Assistant Professor of Pharmacology at the University of Iowa. All of these outcomes satisfy goals of the Career Development Award, i.e., development of independent investigators in the field of breast



cancer research. Because of technical limitations in deriving mammary cell lines overexpressing a growth suppressor, Tasks 1, 3 and 4 will be addressed more fully over the next year. Our studies will provide a key to understanding how Cbl routes EGF receptor to lysosomes for degradation, thereby terminating signaling associated with the development of breast cancer.

## **List of key research accomplishments**

- determined that the recruitment of Cbl to ligand-activated EGF receptor is NOT ligand-selective (Task 1)
- established that EGF-R ubiquitination by Cbl is not sufficient to target the receptor for inactivation and degradation
- determined that EGF-R activated by EGF vs. TGF-alpha is comparably phosphorylated on tyrosine residues (Task 1)
- optimized the experimental conditions for immunofluorescence and downstream signaling experiments to determine whether different EGF-R ligands induce differential regulation of EGF-R subcellular location and signaling (Tasks 5 and 6)

## **List of reportable outcomes**

1. two senior author manuscripts in preparation from the P.I.'s independent laboratory
2. two newly established international collaborations on projects derived from this work
3. presentation of a poster at the American Association for Cancer Research (AACR) meeting, April 2002
4. funded application for a Carver Trust Medical Research Initiative pilot grant
5. funded application for a College of Medicine Research Award pilot grant from the University of Iowa
6. preliminary data obtained for an NIH RO1 grant submission for 06/01/02

## Conclusions

In the past 8.5 months, we have made significant progress with defined aspects of Tasks 1, 5 and 6 of the approved Statement of Work. Other aspects of the Statement of Work will be addressed in the coming year upon establishment of mammary epithelial cell lines stably expressing wild type Cbl. Two attempts to establish expressor lines within the past 8.5 months were unsuccessful. We will attempt this process again, and we will also begin to develop an inducible system for the stable expression of wild type Cbl in mammary epithelial cells. This alternative system may bypass problems caused by the continuous overexpression of wild type Cbl, which has been shown to select for the outgrowth of cell lines that have bypassed Cbl's growth inhibitory mechanisms.

We have determined that the recruitment of Cbl to ligand-activated EGF receptor is NOT ligand-selective (Task 1). EGF-activated EGF-R binds to Cbl and undergoes polyubiquitination, but TGF-alpha-activated EGF-R does the same. Notably, only EGF-activated EGF-R is programmed for inactivation and degradation; TGF-alpha-activated EGF-R is recycled and its signaling is prolonged. Thus, the recruitment of Cbl to EGF-R and its ubiquitination of the receptor are not sufficient to target EGF-R for inactivation and destruction. Other ligand-specified mechanisms are required for this process, and they may be Cbl-dependent.

We also have determined that EGF-R activated by EGF vs. TGF-alpha is comparably phosphorylated on tyrosine residues (Task 1). Again, similar results induced by the two ligands argues that the induction of differential receptor phosphorylation is not the key to the divergent biological impacts of these ligands.

Finally, we have optimized experimental conditions for immunofluorescence and signaling experiments to determine whether EGF-R ligands induce differential regulation of EGF-R subcellular location and signaling (Tasks 5 and 6). These experiments will be undertaken in the immediate future.

**"So what" section** The outcome of our recent investigations contradicts a paradigm in the field: Cbl-mediated EGF-R ubiquitination is not sufficient to target EGF receptors for degradation. Based on these results, we have extended other investigations in the laboratory that are not funded by the DOD award. We have identified Cbl mutants that enhance EGF-R ubiquitination but not EGF-R downregulation or degradation. Using those mutants, we have identified a novel second function of Cbl that controls receptor downregulation and degradation, likely in conjunction with EGF-R ubiquitination. This outcome reveals a new mechanism for the suppression of signaling by EGF-R, a receptor whose deregulation is associated with breast cancer development.

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**Cbl mutants uncouple enhanced EGF receptor ubiquitination from enhanced downregulation and degradation.**

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Signaling through the epidermal growth factor receptor (EGF-R) is critical for normal growth and development, but unattenuated signaling is associated with cellular transformation and tumorigenicity. Cbl, a suppressor of EGF-R signaling, maintains the balance between EGF-R-dependent cell proliferation and differentiation in vivo. Cbl is an E3 protein-ubiquitin ligase that enhances EGF-R ubiquitination, downregulation, and degradation. It has been proposed that receptor ubiquitination may be sufficient to program surface receptors for downregulation and degradation. In structure-function studies, we have identified Cbl mutants that uncouple EGF-R ubiquitination from downregulation and degradation: the mutants enhance EGF-R ubiquitination like wild type Cbl (with similar or delayed kinetics) but are compromised for receptor downregulation and degradation. Our results suggest that a second function, other than E3 activity, is crucial for the suppression of EGF-R signaling by Cbl.

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